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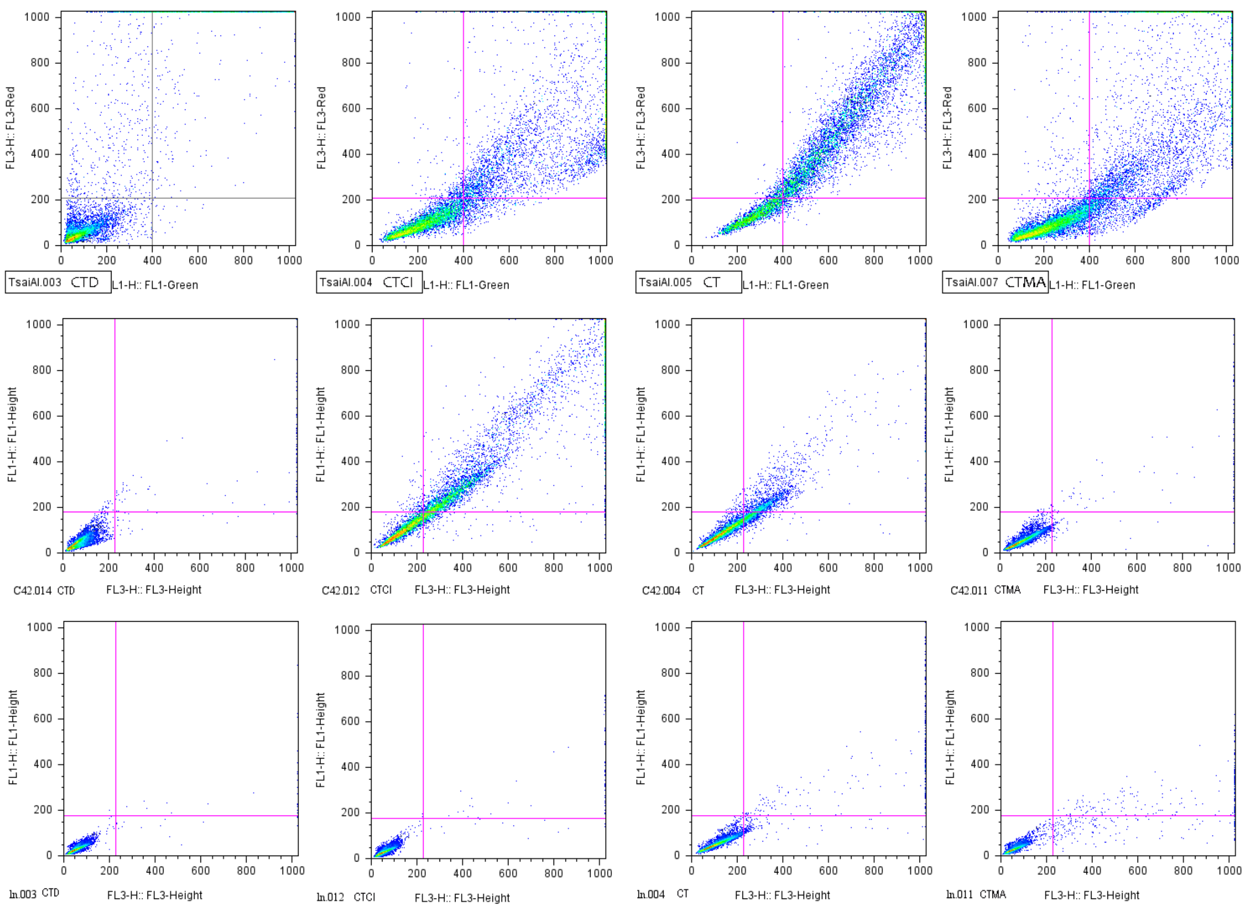
Abstract:
Expression of Chip, a Co-Chaperone Which Interacts with the Androgen Receptor, Results in Loss of AR Expression and Growth Inhibition of Prostate Cancer Cells Waleed Hassen, Xiaoyoung Zheng, Antonio Otero, Erwin Wang, Yuancheng Wang, Sherwin Zargaroff, Jian Pu, Mary Kunjappu, Avrom Caplan and Simon Hall Mount Sinai School of Medicine, New York Introduction and Objectives: Earlier studies in our laboratory and others have demonstrated that over-expression of a co-chaperone, CHIP, an E3 ligase which interacts with HSP-70/90, results in degradation of the androgen receptor (AR) (Cardozo et al, Arch Biochem Biophy, 2003 and He et al, JBC, 2004) Furthermore, it has been shown that CHIP binds directly to a highly conserved sequence on the AR to facilitate degradation. Mutations in this sequence are commonly identified in hormone refractory cancers from TRAMP mice (He et al, JBC, 2004), which result in prostate cancers when over-expressed in a transgenic model (Han et al, PNAS 2005). This suggests that CHIP may have important functions in the homeostatic mechanisms underlying AR function in prostate cancer. These studies explored the outcomes of CHIP over-expression in prostate cancer cells. Methods: CHIP over-expression was achieved by either lipofectamine or adenovirus mediated transduction; the latter contained a tetracycline control mechanism (tet-off). Studies first focused on AR levels by Western blot and effects on growth by either cell counts or MTT assays. Reduction in AR function was addressed by hormone binding studies, while the mechanism of growth suppression was detected by ascertaining cell cycle status via proprium iodide and FACS analysis. Results: By either method, CHIP over-expression resulted in both reduced levels of AR and growth suppression of only AR-expressing cells. Hormone binding studies in AR expressing cells noted reduced levels of hormone binding following CHIP over-expression, correlating with the reduced levels noted by Western blot. CHIP over-expression resulted in growth arrest of hormone sensitive cells while resulting in cell death of hormone insensitive cells. Conclusion: These studies demonstrate that reducing AR levels through manipulation of co-chaperones is an effective method of growth control especially of hormone refractory AR expressing prostate cancer cells.

At the time of filling last year, studies were exploring the mechanism of cell death of AR expressing androgen resistant cell lines. Annexin V FACS analysis had indicated that apoptosis was not a factor in cell death. We first explored a potential role for autophagy, a form of active cell death, by using selective inhibitors of autophagy versus apoptosis. Autophagy is characterized by acidic vesicular organelle (AVO) formation, which is detected and measured by vital staining of acridine orange. Autophagy was inhibited using 1.0 mM 3-MA(4th column, Figure 1), an inhibitor of the phosphatidylinositol 3-phosphate kinase (PI3K), while apoptosis was inhibited by adding CL (2nd column, Figure 1), which is a general caspase inhibitor; both were added to cells the day after infection by Ad-CHIP and Ad-Teton at an MOI of 20. Nonadherent tumor cells and adherent tumor cells that were detached with 0.05% trypsin–EDTA (Invitrogen) were stained with 1.0 µg/mL acridine orange (Sigma-Aldrich) for 15 minutes at room temperature. Stained cells were then analyzed by flow cytometry using the FACScan cytometer (Becton Dickinson, San Jose, CA) and CellQuest software (Becton Dickinson).

Figure 1



As we had noted in earlier studies there was no difference in LNCaP cells (bottom row), comparing active CHIP expression (3rd column) versus control (1st column), indicating no autophagy. In contrast, there were significant differences between columns 1 and 3 in LNCaP-Tsai and C4-2b indicating the potential for cell death via autopagy which was unchanged by inhibiting apoptosis but reduced by inhibiting autophagy. These data were interpreted as indicating that autophagy appeared an important method of cell death following CHIP over-expression in the Ar-expressing hormone refractory cells.

To confirm these findings we choose to examine affected cells at serial time points by electron microscopy following exposure to Ad.CHIP +/- doycysline every 12 hours for 48 hours, given the lack of easy identification by other means of necrotic cell death or autophagy. In LNCap cells we detected a mall population of apoptotic cells in CHIP overexpressing cells above that seen in controls, starting a 36 hours which encompassed ~5-10% of the cells; this would explain the small population of dead cells seen in cell cycle analyses at 36 hours in the first report. Interestingly, in LNCaP-Tsai and C4-2 there was no evidence of lysosomal congregration, the hallmark of autophagy, rather vaculolization of cytoplasm and disintergration of cellular membranes, both consistent with necrotic death – this we believe is the central cause of death in AR-expressing hormone refractory cells following inactivation of AR function via CHIP over-expression. These findings are being prepared in manuscript form for submission later this year.

These results confirmed our earlier findings and supported our hypothesis that AR expressing androgen independent prostate cells are dependent or AR for survival and not just for growth, like androgen sensitive cells. Therefore, in a departure from the SOW we sought identify the genes, either turned on or off, which were responsible for this finding following CHIP expression.

Methods:

Affymetrix arrays. LNCaP, C42 and LNCaP-Tsai cells were harvested 24h after adenovirus infection (MOI = 20). Total RNA was isolated from the indicated cell lines using the RNeasy Mini kit (QIAGEN Inc., Chatsworth, California, USA) according to the manufacturer's protocol The protocol for mRNA quality control and gene expression analysis was that recommended by Affymetrix (Santa Clara, California, USA). In brief, approximately 1 μg of mRNA was reverse transcribed with an oligo(dT) primer that has a T7 RNA polymerase promoter at the 5′ end. Second-strand synthesis was followed by cRNA production incorporating a biotinylated base. Hybridization to Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array overnight for 16 hours was followed by washing and labeling using a fluorescently labeled Ab. The arrays were read and data processed using Affymetrix equipment and software.

Data Analysis. Raw data from CEL files were normalized by robust GC-RMA method and then experimental batch effect was adjusted by empirical Bayes method (Johnson et al. 2006)[1]. The expression value for a gene with multiple probe sets was given by median expression value of these probe sets. Differentially expressed genes were identified by LIMMA method (linear models for microarray data) (Smyth et al. 2004)[2] and fold change. Cutoffs of FDR adjusted p<=0.01 and 4-fold change were used to select significant genes. All computations were done in statistical package R with corresponding libraries from Bioconductor (www.bioconductor.org). To better understand gene expression profiles in the context of pathways, biological functions and gene interaction networks, differentially expressed genes were further annotated and analyzed through DAVID (Dennis et al. 2003)[3] and Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). First we compared the different expression between CHIP expression group and no CHIP expression in each cell lines; then we compared the different expression between androgen dependent and androgen impendent cell lines.

To identify CHIP-regulated genes, we infect androgen dependent LNCap cell and androgen independent C42b and Tsai cell lines with Adenovirus which conditionally express CHIP and used Affymetrix microarrays to screen for CHIP-regulated transcripts. Selected genes were validated by Q-RT-PCR. The results reveal that the downstream targets of CHIP include genes that are implicated in cell cycle progression, regulation of cell proliferation and apoptosis (e.g., RND3, ARC, edg-4). These results suggest that CHIP plays a novel role in cell homeostasis.

1. CHIP-induced alteration of gene expression was examined using cDNA microarray assay.
   A. Genes' expression induced by CHIP expression in LNCap-Tsai and LNCap C42b cells but not in LNCap.

GenBank	Gene.Symbol	Tasi LogRatio	C42B LogRatio
AL833762	DKFZp666G057	-5.0855	-4.7688
AI018322	PLAC8L1	-3.0452	-2.8869
BF968097	---	-3.3016	-2.6168
BE463997	ARL9	-4.6413	-3.5082
AB018333	SASH1	-2.8681	-2.4297
AA129774	LOC400793	-3.3986	-2.0182
BG540494	PALM2-AKAP2	-3.8419	-3.4354
BG054844	RND3	-3.9733	-4.7805
AA018968	PIK3R1	-2.1466	-2.7619
AI967987	MUM1L1	-4.2419	-3.1636

   B. Genes' expression decreased by CHIP expression in LNCap-Tsai and LNCap C42b cells but not in LNCap.

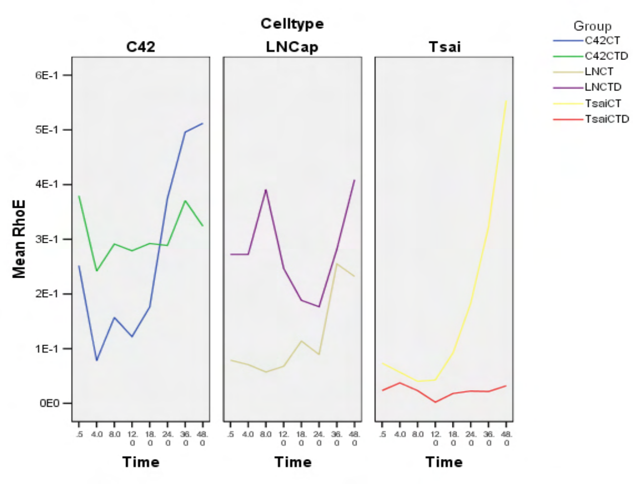
GenBank	Gene.Symbol	Tasi LogRatio	C42B LogRatio
NM_001543	NDST1	3.06	3.02
Z83838	ARHGAP8 /// LOC553158	2.32	2.75
AI570493	LOC283377	3.28	3.42
AV734843	FLJ22833	2.88	2.39
AK001080	WDR6	2.74	2.46
L11669	TETRAN	2.44	2.08
AI093963	EID-3	4.23	5.10
NM_001517	GTF2H4	2.63	2.03
NM_018056	TMEM39B	2.12	2.27
AW135740	FLJ36812	2.62	2.92
AL530748	GEMIN7	2.62	3.71
AB028127	PIGM	2.80	2.40
AI912351	NOL3 (ARC)	2.55	2.00
NM_024509	LRFN3	2.89	2.04
AA150455	KIAA0276	2.03	4.05
NM_001105	ACVR1	2.46	2.90
BF345728	LOC147727	2.04	2.51
NM_003536	HIST1H3H	2.84	3.57
AW467472	APPL	2.62	2.06
AB037853	KIAA1432	2.44	2.28
AL531790	MGC88387	3.03	2.13
AW002273	FBXL17	2.71	2.82
AI681419	LOC388327	2.26	3.03
BC005810	CLEC11A	2.15	3.51
AI264247	ATP1A1	2.32	2.17
AF131747	KIAA0830	2.87	2.30
BC001428	PLEKHB2	2.14	2.23
NM_005227	EFNA4	3.73	3.44
NM_004085	TIMM8A	2.14	2.31
NM_022492	FLJ12788	2.35	2.34

BE740761	HIST1H4H	2.16	2.73
BE326857	CYP4V2	3.04	2.75
BF977145	C1orf85	4.16	2.25
AK024446	ABCC10	3.09	2.92
BE042976	MGC17330	2.18	3.04
AL122088	LYSMD1	2.40	3.54
X76775	HLA-DMA	2.83	2.67
AI431931	GIMAP2	2.57	2.60
BF939830	LOC254128	3.13	3.35
AW080835	C1orf51	2.76	2.72
NM_006858	TMED1	2.01	2.47
BE467260	DCBLD1	2.44	2.84
AF011466	EDG4	2.38	2.16
NM_019082	DDX56	2.08	3.60

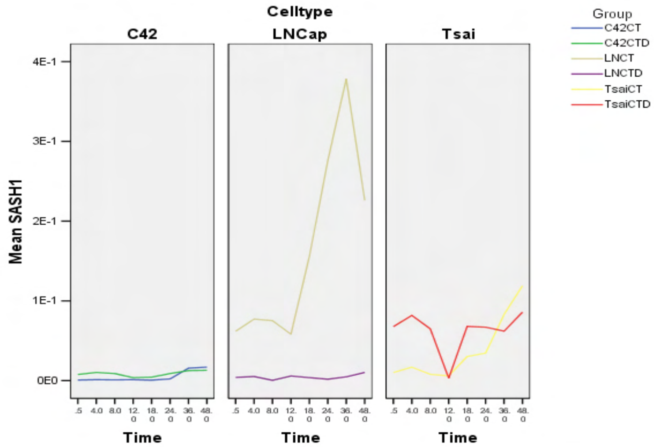
Quantitative RT-PCR. Cells were harvested at 0.5h, 4h, 8h, 12h, 16h, 24h, 36h, 48h after adenovirus infection (MOI = 20). cDNAs were synthesized from 1µg total RNA using Omniscript RT kit (Qiagen). Aliquots of cDNA were amplified in a LightCycler 480 Real-Time PCR System (Roche Applied Science) using SYBR green qPCR Master Mix (Roche Applied Science) in triplicate reactions. PCR amplification was performed with the gene-specific primers listed in Supplemental Table 1. PCR cycling conditions were: 95 °C for 15 min and 45 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min. Transcript levels were calculated relative to β - actin mRNA levels as endogenous control. Relative expression was calculated.

2. Validation of CHIP regulated changes of genes expression using a Q-RT-PCR–based assay.

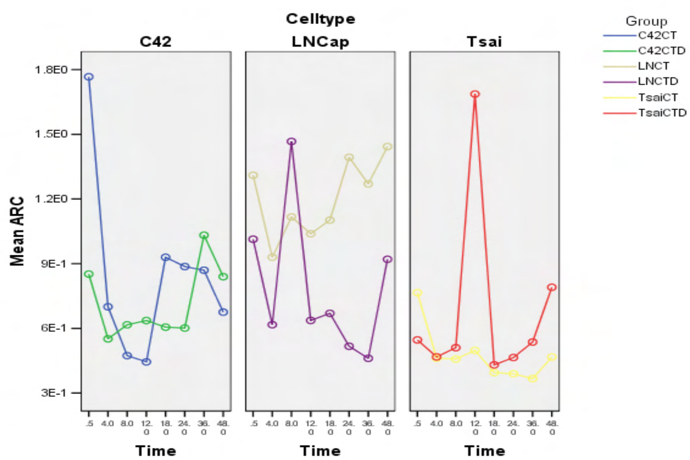
A. CHIP gene expression induced RND3 (RhoE) significantly expression in LNCap Tsai and LNCap C42 but not in LNCap cells. Others papers also show that RhoE may inhibit cell cycle progression and Ras-induced transformation.[4] Forced RhoE overexpression in a prostate cancer cell line inhibits the expression of two proteins essential for G2/M transition, namely CDC2 and cyclin B1, and induces G2/M arrest. [5]



B. CHIP gene expression only induced SASH1 gene expression in LNCap but not in LNCap Tsai and LNCap C42 cells. *SASH1* has been implicated to act as a tumour suppressor gene in human breast cancer[6]. Sash1 has an SH3 region together with two SAM domains suggest that it is also involved in signal transduction.

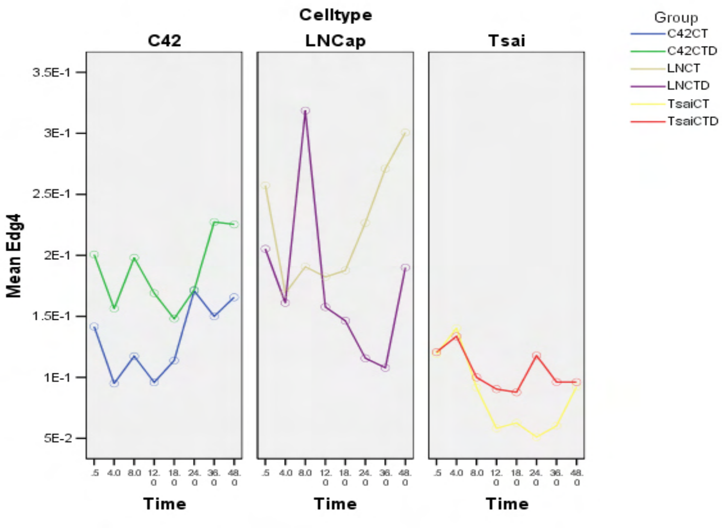


C. CHIP gene expression lead to significantly elevation of **ARC** expression only in LNCap cells



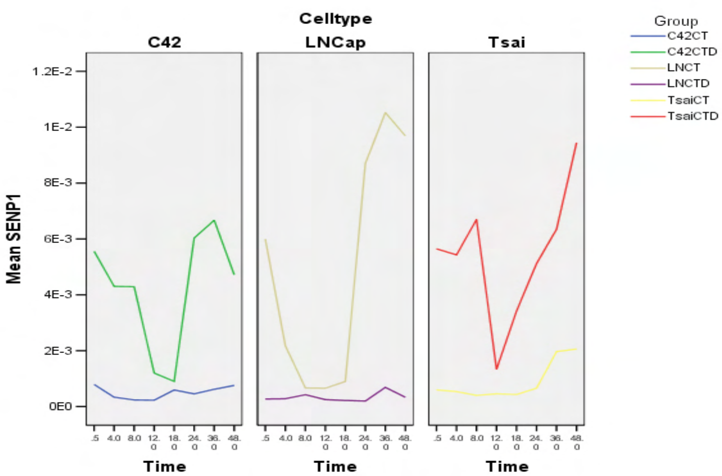
CHIP gene expression lead to significantly elevation of **Edg4** expression in LNCap cells, but to reduction of Edg4 expression in LNCap Tsai and LNCap C42 cells.

Lysophosphatidic acid (LPA) is an endogenous lipid growth factor that is thought to play important roles in cell proliferation and antiapoptosis and therefore may have roles in the development and progression of benign prostatic hyperplasia (BPH). Most of these responses are mediated through cell surface-specific receptors, which belong to the endothelial differentiation gene (Edg) family of G protein-coupled receptors. Cognate LPA-specific receptors Edg-2, Edg-4, and Edg-7 are now called LPA1, LPA2, and LPA3, respectively.[7] LPA elicits a variety of cellular responses, which include mitogenic effects on the cell cycle, induction of cancer cell invasion, regulation of actin stress fiber formation and focal adhesion assembly, cell motility, and mobilization of intracellular calcium.[8]



D. CHIP gene expression lead to significantly elevation of **SENP1** expression in LNCap cells, but to significantly reduction of SENP1 expression in LNCap Tsai and LNCap C42 cells.

The induction of SENP1 is observed with the chronic exposure of prostate cancer cells to androgen and/or interleukin (IL) 6. SENP1 upregulation modulates the transcriptional activity of androgen receptors (ARs) and c-Jun, as well as cyclin D1 expression. Initial in vivo data from transgenic mice indicate that overexpression of SENP1 in the prostate leads to the development of prostatic intraepithelial neoplasia at an early age. Collectively, these studies indicate that overexpression of SENP1 is associated with prostate cancer development. [9]



Conclusions:

It appears that a variety of important genes are either over or underexpressed in cells which die following CHIP expressing. SENP1 appears to be a most attractive target for further evaluation.

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